Latent effects from low salinity stress and the interactive influence of temperature and salinity on larval and juvenile growth in the marine gastropod *Crepidula fornicata*

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Abstract

Sea surface temperatures have been rising and are predicted to continue rising in coming years because of global warming. In addition, salinity has been decreasing in high latitudes and is expected to continue decreasing due to altered precipitation patterns and glacial melting caused by climate change. Many marine organisms that are adapted to the present conditions may be drastically affected by these oceanic changes. Early life stages (larvae and juveniles) will be especially susceptible, since they do not yet have fully developed adult defenses. In this study, we investigated the effects of reduced salinity (20 psu and 30 psu control) and altered temperature (15, 20, 25, and 29°C) on the growth, percent inorganic content (representing calcification), and feeding rates of juveniles and larvae of the invasive intertidal snail *Crepidula fornicata*. Both larval and juvenile growth rates were significantly depressed by low salinity and elevated by higher temperatures. The salinity that snails were exposed to as larvae significantly impacted their juvenile growth rates in 4 out of 6 experiments, an example of latent effects, but the magnitude and direction of this effect depended on temperature and parentage. In addition, juvenile feeding rates were significantly depressed in low salinity but there was little change in percent inorganic content. Larval percent inorganic content at 20°C was 27% lower in 20 psu than in 30 psu. In conclusion, *C. fornicata* may experience more favorable conditions in a warmer future: growth rates will increase, probably making juveniles more resistant to predation. However, in regions where salinity is decreasing, *C. fornicata* juveniles will likely grow and feed more slowly, thereby increasing predation risk by forcing them to spend more time at more vulnerable smaller sizes. Thus, the future of *C. fornicata* and their potential to continue invading new habitats will depend greatly on the specific salinity and temperature conditions they will be exposed to throughout larval and juvenile development.

Introduction

Anthropogenic climate change has caused global mean air temperature over the past 3 decades to rise to higher levels than in any other decades on record (IPCC 2013). In consequence, sea surface temperatures have been increasing at the alarming average rate of 0.11°C per decade since at least 1971 (IPCC 2013). In addition, rising temperatures have altered precipitation patterns and increased the rate of glacial melting, causing salinity to decrease in high latitudes since the 1950s (IPCC 2013). While these salinity changes are not large in magnitude (~0.5 psu over 50 years along the Eastern US Coast [IPCC 2013]), they could cause the frequency or duration of salinity fluctuations to increase in near-shore ecosystems like estuaries, where salinity can drop by 33-67% in a matter of hours (Chaparro *et al.* 2008, Khangaonkar *et al.* 2011). For example, salinity fluctuations in the Puget Sound of Washington state have been increasing in severity and frequency in recent years (Sophie George, personal communication). These documented changes are expected to continue in the future, or worsen, with potentially drastic consequences for marine ecosystems. Species ranges will shift (Scavia *et al.* 2002, Harley *et al.* 2006), some species will go extinct (Thomas *et al*. 2004), others will persevere (Moritz and Agudo 2013), and invasive species may even do better (Stachowicz *et al*. 2002). The only inevitable fact is that biological responses to multi-faceted climate change will be incredibly complex and difficult to predict without extensive basic research.

Especially affected by climate change will be the early life stages of marine organisms which lack the fully developed defenses of the adult (Gosselin and Qian 1997). These early life stages also have a huge influence on the future distribution and abundance of adult populations, which are often limited by recruitment, a product of larval supply and juvenile survival (Keough and Downes 1982, Hunt and Scheibling 1997). Both larvae and juveniles are subject to

extremely high mortality rates, reaching as high as 87% over 2 minutes in ascidian larvae (Olsen and McPherson 1987) and over 90% for juveniles of a number of species in the first few days or weeks after metamorphosis (reviewed in Gosselin and Qian 1997). With such high background mortality, any additional disturbance caused by anthropogenic temperature or salinity changes could push recruitment below required minimum levels for population sustainability. Thus, it is vitally important to understand how these early life stages will be affected by climate change in order to predict how adult populations will be affected.

In addition to direct mortality, the non-lethal effects of climate change could be just as devastating. Juveniles (Vermeij 1972, Paine 1976) and possibly larvae (Rumrill 1990, Pechenik 1999, Morgan 1995) are more vulnerable to predation and environmental stresses at smaller sizes, so decreased growth may indirectly increase mortality by making larvae and juveniles spend more time in the more vulnerable smaller sizes and, in the case of larvae, forcing them to spend more time in the plankton subject to pelagic predation (Rumrill 1990). Also, if larvae spend more time in the plankton or their swimming abilities are impaired by temperature or salinity changes, their dispersal and therefore their chance of ending up in appropriate habitat would likely be affected, which could reduce the supply of larvae to adult populations.

Another non-lethal effect of salinity change could be a reduction in calcification in calcifying larvae and juveniles since low salinity seawater, with a lower concentration of calcium, is known to slow calcification in such calcifying invertebrates as adult mussels (Maloney and Dodd 1967) and brittle stars (Donachy and Watabe 1986). Therefore, these individuals may take even longer to fully develop adult defenses, many of which are dependent on calcification.

Further complicating the biological response to climate change is the phenomenon of latent effects, wherein stresses experienced during the larval stage impact juvenile performance or growth (Pechenik *et al.* 1998, Pechenik 2006). Latent effects have been documented following larval exposure to low food concentration (snails: Pechenik *et al.* 1996a, 1996b), ocean acidification (oysters: Hettinger *et al.* 2012), low salinity (polychaetes: Pechenik *et al.* 2001, barnacles: Thiyagarajan *et al.* 2007), and delayed larval metamorphosis (bryozoans: Woollacott *et al.* 1989, barnacles: Pechenik *et al.* 1993, ascidians: Marshall *et al*. 2003). Therefore, it is not sufficient to only consider the conditions experienced by juveniles when attempting to predict their response to environmental changes; the conditions experienced as larvae must be considered as well.

One widespread, calcifying, osmoconforming, marine invertebrate likely to be affected by climate change is the marine gastropod *Crepidula fornicata*. *C. fornicata* is native to the Eastern U.S. coast and an extremely successful invader of Northern Europe, Japan, and the Western U.S. coast (Blanchard 1997). Females release free-swimming, shelled veliger larvae in hatches usually fathered by multiple males (Dupont *et al*. 2006). The larvae then spend anywhere from 7-27 days in the plankton before settling and metamorphosing (Pechenik 2006). *C. fornicata* is an ideal species for the study of early life stages since the juveniles and larvae can be reared in the lab with very low mortality and once metamorphic competence is reached, larvae can easily be induced to metamorphose with a simple chemical (Pechenik and Gee 1993, reviewed by Henry *et al.* 2010). In addition, by studying the effects of climate change on this invasive species we may be able to predict how native and invasive populations will be affected, where *C. fornicata* will be able to invade in the future, and how related molluscs will be affected.

Previous research has demonstrated that *C. fornicata* juveniles can separately tolerate salinities as low as 15 psu and temperatures as high as 32°C (Pechenik and Eyster 1989) while larvae can tolerate salinity as low as 15 psu (Diederich *et al.* 2011) and temperatures up to at least 29°C (Pechenik and Lima 1984). A number of studies have documented that *C. fornicata* larvae grow faster at higher temperatures (Pechenik 1984, Pechenik and Lima 1984, Pechenik and Heyman 1987, Klinzing and Pechenik 1990). For example, Klinzing and Pechenik (1990) found that larvae grew more than twice as fast in terms of shell length at 25°C than at 16°C. Another study discovered that *C. fornicata* larvae stopped growing and fed at only 25-50% of control rates during exposure to low salinities of 15 and 20 psu and that juveniles transferred to 15 psu for 12-48 hours and then returned to normal salinity grew 50% slower than unstressed controls for the 3 days following the salinity shock (Diederich *et al.* 2011). But no studies have investigated the interactive effects of temperature and salinity on *C. fornicata* larval or juvenile growth. Also, while many studies have looked at the effects of salinity or temperature on growth in terms of shell length, no studies have yet investigated the effects on relative shell/tissue growth, which may be affected by salinity change because of the increased difficulty of calcifying at low salinity, as described above.

Finally, one previous study found no evidence in *C. fornicata* for latent effects of larval exposure to low salinity on juvenile growth (Diederich *et al*. 2011). However, after metamorphosis these juveniles were all reared in stress-free, normal salinity conditions. No studies have investigated the effect of larval exposure to low salinity on juvenile performance in stressful (low salinity) conditions, or how this effect is modulated by temperature.

In this study, we sought to answer the following questions: 1) What are the interactive effects of salinity and temperature on *C. fornicata* larval and juvenile growth? 2) How does

larval exposure to low salinity impact juvenile growth, and how is this effect modulated by a) temperature or b) the salinity juveniles are reared in? and 3) Are reduced juvenile growth rates in low salinity caused in part by reduced feeding rates? To address these questions, we measured the growth (both in terms of shell length and shell/tissue mass) of juveniles and larvae in a number of temperature and salinity treatments, we investigated the effects of larval exposure to low salinity on subsequent juvenile growth both in low and normal salinity and at 3 different temperatures, and we measured feeding rates in low and normal salinity of juveniles reared in low or normal salinity to determine a possible cause of reduced growth rates and whether juveniles can acclimate to low salinity.

Materials and methods

Adult collection and maintenance

Adult *C. fornicata* were collected from Nahant, MA in September 2012, September 2013, and January 2014 and maintained in the lab until larvae were released. Adults collected in January were slowly acclimated to lab temperature (about 23°C) to prevent mortality from heat stress. All adults were kept in a mixture of Instant Ocean artificial seawater (ASW) and natural seawater at approximately 30 psu and they were regularly fed the microalgae *Isochrysis galbana* and *Dunaliella tertiolecta*. Released larvae were collected on a 130 µm mesh, transferred to 1-gallon glass jars half-filled with aerated 0.45 µm filtered seawater (FSW), and fed *I. galbana* until needed for experiments.

Larval and juvenile maintenance

Larvae to be used in experiments were kept in glass bowls filled with 40-50 ml FSW, adjusted to the appropriate salinity with deionized water. Larvae were fed *I. galbana* at

approximately 1.8×10^5 cells/ml. Temperature was maintained with incubators that were set to a 12light/12dark cycle.

When larvae were determined competent to metamorphose (see below), they were transferred to ASW with 20 mM excess KCl to induce metamorphosis (Pechenik and Heyman 1987, Pechenik and Gee 1993). Juveniles were reared exactly as described for larvae except they were kept in 50% FSW and 50% ASW, adjusted to the appropriate salinity with deionized water. Juveniles were fed *Isochrysis galbana* and occasionally *Dunaliella tertiolecta* at 1.8×10^5 cells/ml. Food concentrations did not drop below 7×10^4 cells/ml, where feeding rates begin to decrease (Pechenik and Eyster 1989).

Water was changed every 2-3 days. Larvae were transferred to new, clean bowls at each water change. Juvenile bowls were cleaned and refilled with fresh water. Salinity was confirmed with a hand-held refractometer.

Growth measurements

Shell length measurements were obtained for larvae and juveniles using a dissecting microscope fitted with an ocular micrometer. Larvae were measured at magnifications of 50 or 63X and juveniles were measured at 16-63X, depending on their size. The longest shell length was always recorded and used for subsequent growth calculations.

Additionally, organic (tissue) and inorganic (shell) masses were measured for both larvae and juveniles. Juveniles were measured individually while larvae were measured 5-9 to a sample in order to get accurate measurements despite their small size. All masses were measured with a Mettler Toledo MT5 balance to the nearest 0.001 mg. Samples were quickly rinsed in deionized water to remove any adhering salt or formalin, placed on pre-weighed foil, dried in a drying oven at approximately 55°C for at least 48 hours, and weighed to obtain the total dry mass. Then,

samples were combusted at 500°C for at least 12 hours to burn off all organic matter, returned to the drying oven for at least 1 hour, and weighed again to obtain the inorganic mass. Foil blanks put through the same process did not change mass by more than 13 µg. Relative inorganic content was calculated by dividing inorganic mass by total dry mass.

Growth rate experiment 1 – The long-term effects of temperature and salinity on juvenile growth

This experiment was conducted on juveniles reared from larvae released in 2 separate hatches in September 2012. Juveniles were split into treatments as soon as they had metamorphosed. There were 6 treatments, comprising every combination of the temperatures 15, 20, and 25°C and the salinities 20 and 30 psu, and each treatment had 12 replicate juveniles. Juveniles were measured every 2-4 days until they reached approximately 6 mm in length, at which point they were sacrificed for organic/inorganic mass determination. Cumulative growth rates were calculated for each day juveniles were measured by subtracting initial size from the size at that day and dividing by the number of days elapsed.

Growth rate experiment 2 – The effects of larval exposure to low salinity on juvenile growth

Experiments were started 1-4 days after hatching. First, 12 larvae were measured from each hatch to get initial shell lengths, then larvae were distributed into a total of 6 treatments consisting of all combinations of the temperatures 20, 25, and 29°C and the salinities 20 and 30 psu (Fig. 1). Each treatment had 4 replicate bowls with approximately 15 larvae per bowl filled with ~50 ml algal suspension. Three replicates were measured for larval growth rates and a 4th was used to test for metamorphic competence.

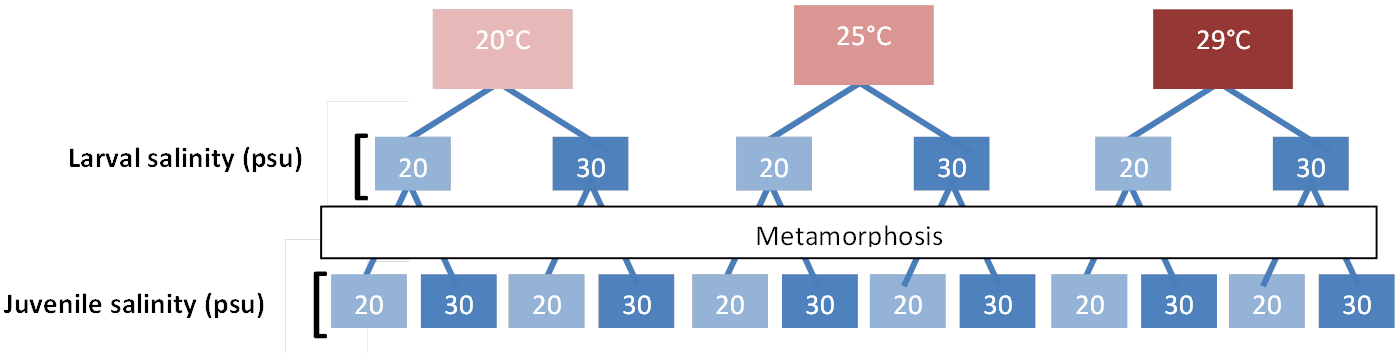

Figure 1. Experimental design for juvenile growth experiment 2.

Larval shell lengths were determined periodically a minimum of 3 times before metamorphosis. When larvae were close to 800 µm long, 7-8 larvae from the 4th replicate were tested for competence by transferring them to ASW + 20mM KCl for 6 hours. If at least 75% metamorphosed, larvae from that treatment were determined competent to metamorphose. Or, if larvae were larger than 800 µm before testing for competence, they were assumed to be competent (Pechenik and Heyman 1987). Once competent, larvae from replicates 1, 2, 3, and any from replicate 4 that had not previously been exposed to KCl were transferred to ASW + 20 mM KCl for 6 hours to induce metamorphosis (Pechenik and Heyman 1987).

After larvae metamorphosed into juveniles, each larval treatment was split into 2 new salinity treatments: 20 and 30 psu, with 12 replicate juveniles per treatment (Fig. 1). Temperature was kept constant for the entire experiment. Thus, there were a total of 12 different juvenile treatments (3 temperatures x 2 larval salinities x 2 juvenile salinities). Juveniles were initially measured 1-2 days after metamorphosis, then every 4-5 days after that. Once juveniles reached 4-4.5 mm in length, they were sacrificed for organic/inorganic mass measurements.

Experiments were conducted on a total of 3 larval hatches, with 2 temperature treatments per hatch. The 29°C treatments were run once, the 25°C treatments thrice, and the 20°C treatments twice. Each of these 6 experiments was analyzed as a separate experiment.

Larval inorganic content experiment

An experiment was conducted to determine the effects of salinity and temperature on larval relative inorganic content. Treatments consisted of the temperatures 20 and 25°C and the salinities 20 and 30 psu. Each treatment had 5 replicate bowls with 15 larvae per bowl in ~50 ml algal suspension. Larvae were put into treatments 2 days after hatching. First, 12 larvae were measured from each hatch to get initial shell lengths. At the end of the experiment, all larvae in the first 3 replicates were measured. Once larvae reached about 800 µm in length, the approximate size of metamorphic competence (Pechenik and Heyman 1987), all larvae from the 5 replicates (~75 total) were fixed and used for inorganic/organic mass measurements. Larvae were fixed in 10% buffered formalin for a minimum of 30 minutes and then processed as described above for organic/inorganic mass measurements.

Feeding rate experiment

Two feeding rate experiments were conducted to determine whether juveniles feed more slowly in low salinity and if they can acclimate to low salinity. Larvae reared in the lab at room temperature (~23°C) and normal salinity (~30 psu) were induced to metamorphose with KCl when competent and divided in half immediately after metamorphosis into 2 treatments at 20 or 30 psu. Juveniles were reared in one large container (~3-4 L, bubbled vigorously) per treatment and fed *I. galbana* and *D. tertiolecta*.

Feeding rate experiments were conducted on juveniles in each treatment when they were approximately the same size range, so the experiment on juveniles reared in 30 psu was conducted 20 days after metamorphosis, while the experiment on juveniles reared in 20 psu was conducted 26 days after metamorphosis in order to allow the 20 psu reared juveniles time to reach a similar size.

Feeding rate experiments were conducted in 12-well plates filled with 5 ml *I. galbana* suspension at approximately 1.8×10^5 cells/ml and the appropriate salinity. Each feeding rate experiments had 2 treatments at 20 psu and 30 psu and there were 17 juveniles and 3 control wells (filled only with the algal suspension in order to account for the passive change in particle density) per treatment. Control wells were treated exactly the same as experimental wells. Juveniles were first allowed to pre-feed in their well for 2 hours. Then the old water was dumped out, the well was rinsed with filtered water, and then refilled with 5 ml new algal suspension. Three 1 ml samples were taken of the new water at each salinity before it was distributed to juveniles and counted with a Coulter Counter to determine initial cell concentration. After juveniles had fed for 2 hours, each well was stirred by pipetting 1 ml in and out twice and then 1ml samples were taken from each well. These samples were counted on the Coulter Counter and clearance rate (CR; ml/hour) was calculated from the following formula after Coughlan (1969):

$$CR = \frac{V\left(\ln(C_i) - \ln\left(C_f\right) - a\right)}{t}$$

where V=volume (5ml), t=time in hours, C_i=initial cell concentration, C_f=final cell concentration, and $a = \frac{\ln(C_i) - \ln(C_f\prime)}{t}$ where $C_f{}'$ is the average final cell concentration of the control wells.

Statistical analyses

All statistical tests were performed with Graphpad Prism 6. For the 1st growth rate experiment, a 2-way ANOVA was run on juvenile growth rates from day 1 to day 33 (the final day at which all treatments were measured) to determine the effects of salinity and temperature and a 1-way ANOVA followed by a Bonferroni post-hoc test was run on the day 33 shell lengths of each of the 6 treatments to determine whether the treatments had significantly diverged in size. In addition, a 2-

way ANOVA was run on arcsine-transformed relative organic contents to determine the effects of salinity and temperature.

For the 2nd growth rate experiment, larval shell growth rates were calculated from changes in shell length from the start of the experiment to the day they were induced to metamorphose, while juvenile growth rates were calculated from changes in shell length from day 1 or 2 after metamorphosis to day 16 after metamorphosis. Larval growth rates from each of the 3 larval hatches were analyzed with 2-way ANOVAs to determine the effects of temperature and salinity. Juvenile growth rates were analyzed with 2-way ANOVAs run on each of the 6 experiments (corresponding to the 6 temperature treatments) followed by Tukey post-hoc tests. The ANOVAs tested the effects of the factors larval salinity and juvenile salinity. Juvenile relative inorganic content was analyzed the same way after data were arcsine transformed.

Larval relative inorganic content was first arcsine transformed, then analyzed with a 2-way ANOVA to determine the effects of temperature and salinity.

For each feeding rate experiment, juvenile size and feeding rate were analyzed with t-tests to ensure juveniles in each feeding treatment were the same size and determine if salinity affected feeding rate.

Results

Growth rate experiment 1: The long-term effects of temperature and salinity on juvenile growth

A few juveniles (8.3% of total) had perished by the end of the 33-day experiment; most of these deaths were due to suicide (juveniles crawled out of the water) or experimental error.

Table 1. Summary of 2-way ANOVAs performed on larval and juvenile growth and relative inorganic masses. Larval salinity is the salinity in which larvae were reared while juvenile salinity is the salinity in which juveniles were reared. Highlighted cells indicate significant differences, stars represent the degree of significance, and "ns" means "not significant."

			Larval salinity		Juvenile salinity		Temperature		Interaction	
Measurement	Larval batch	Temperature treatments (°C)	ANOVA F (df)	p-value	ANOVA F (df)	p-value	ANOVA F (df)	p-value	ANOVA F (df)	p-value
Larval growth	1	20, 25	24.47 (1,8)	0.0011 (**)			35.69 (1,8)	0.0003 (***)	2.186 (1,8)	0.1776 (ns)
	2	25, 29	300.2 (1,8)	<0.0001 (****)			15.92 (1,8)	0.0040 (**)	10.98 (1,8)	0.0106 (*)
	3	20, 25	0.8937 (1,8)	0.3721 (ns)			333.3 (1,8)	<0.0001 (****)	17.73 (1,8)	0.0030 (**)
Juvenile growth	1	20	55.73 (1,38)	<0.0001 (****)	60.69 (1,38)	<0.0001 (****)			2.231 (1,38)	0.1435 (ns)
	1	25	0.6530 (1,41)	0.4237 (ns)	24.41 (1,41)	<0.0001 (****)			0.7272 (1,41)	0.3988 (ns)
	2	25	9.172 (1,39)	0.0043 (**)	20.09 (1,39)	<0.0001 (****)			8.401 (1,39)	0.0061 (**)
	2	29	2.321 (1,40)	0.1355 (ns)	20.88 (1,40)	<0.0001 (****)			2.297 (1,40)	0.1374 (ns)
	3	20	4.974 (1,42)	0.0311 (*)	70.56 (1,42)	<0.0001 (****)			0.7129 (1,42)	0.4033 (ns)
	3	25	4.687 (1,45)	0.0357 (*)	76.86 (1,45)	<0.0001 (****)			0.03066 (1,45)	0.8618 (ns)
	4	15, 20, 25			100.7 (1,60)	<0.0001 (****)	101.4 (2,60)	<0.0001 (****)	4.806 (2,60)	0.0116 (*)
Larval relative inorganic content	5	20, 25	9.585 (1,39)	0.0036 (**)			1.020 (1,39)	0.3186 (ns)	32.02 (1,39)	<0.0001 (****)
Juvenile relative inorganic content	1	20	4.091 (1,33)	0.0513 (ns)	0.09061 (1,33)	0.7653 (ns)			11.88 (1,33)	0.0016 (**)
	1	25	40.73 (1,43)	<0.0001 (****)	0.8137 (1,43)	0.3720 (ns)			0.6432 (1,43)	0.4270 (ns)
	2	25	2.828 (1,36)	0.1013 (ns)	1.855 (1,36)	0.1817 (ns)			1.351 (1,36)	0.2528 (ns)
	2	29	3.010 (1,32)	0.0924 (ns)	12.72 (1,32)	0.0012 (**)			5.641 (1,32)	0.0237 (*)
	3	20	0.8440 (1,39)	0.3639 (ns)	33.62 (1,39)	<0.0001 (****)			0.4478 (1,39)	0.5073 (ns)
	3	25	0.05392 (1,41)	0.8175 (ns)	15.99 (1,41)	0.0003 (***)			0.08770 (1,41)	0.7686 (ns)
	4	15, 20, 25			1.692 (1,53)	0.1990 (ns)	17.48 (2,53)	<0.0001 (****)	4.916 (2,53)	0.0110 (*)

Differences in juvenile size and growth rates between all 6 treatments (larval salinity was not manipulated in this experiment) were apparent by day 13 after metamorphosis (Fig. 2). Juveniles continued growing in all experiments for the duration of the experiment, even at 15°C in low salinity (20 psu), and shell growth rate gradually increased for animals in most treatments

over the duration of the experiment (Fig. 2). However, 2 exceptions are apparent to this rule, both relating to juveniles reared at 25°C. Firstly, juveniles reared at 25°C and 20 psu that initially had similar growth rates to juveniles reared at 25°C and 30 psu, slowed their growth dramatically after day 7 (Fig. 2A). The growth rates of the individuals reared at 25°C and 20 psu decreased rapidly for the next week before beginning to gradually increase as in the other treatments. Secondly, the shell growth rates of juveniles reared at 25°C and 30 psu increased until day 17 but then decreased for the remainder of the experiment (Fig. 2B).

Low salinity significantly depressed juvenile growth rates (2-way ANOVA, $F=100.71$, $df=1,60$, $p<0.0001$) and high temperatures significantly increased juvenile growth rates (2-way ANOVA, $F=101.37$, $df=2,60$, $p<0.0001$). In addition, the interaction of temperature and salinity was significant (2-way ANOVA, $F=4.81$, $df=2,60$, $p=0.0116$), indicating that salinity had a more pronounced effect on growth rates at higher temperatures (Fig. 2, Table 1). Juveniles in the low salinity treatments generally experienced reduced growth rates between days ~8-15 followed by increasing growth rates for the remainder of the experiment. Juveniles reared at the highest temperature in full-strength salinity (25°C 30 psu) grew about 4 times faster than those reared at the lowest temperature in reduced salinity (15°C 20 psu; Fig. 2).

Growth rate experiment 2 – The effects of larval exposure to low salinity on juvenile growth

Mortality

Larval mortality in all experiments was 0% or very close to 0%. The only exception was one replicate of the 25°C, 30 psu treatment with individuals from larval hatch #2, which was infected by a red growth that killed all but 4 larvae (73% mortality) in that replicate. Juvenile mortality was also very low, and any juvenile deaths were usually due to suicide or experimental

error as in experiment 1. Overall juvenile mortality rates were 14% for larval hatch #1, 16% for larval hatch #2, and 5% for larval hatch #3.

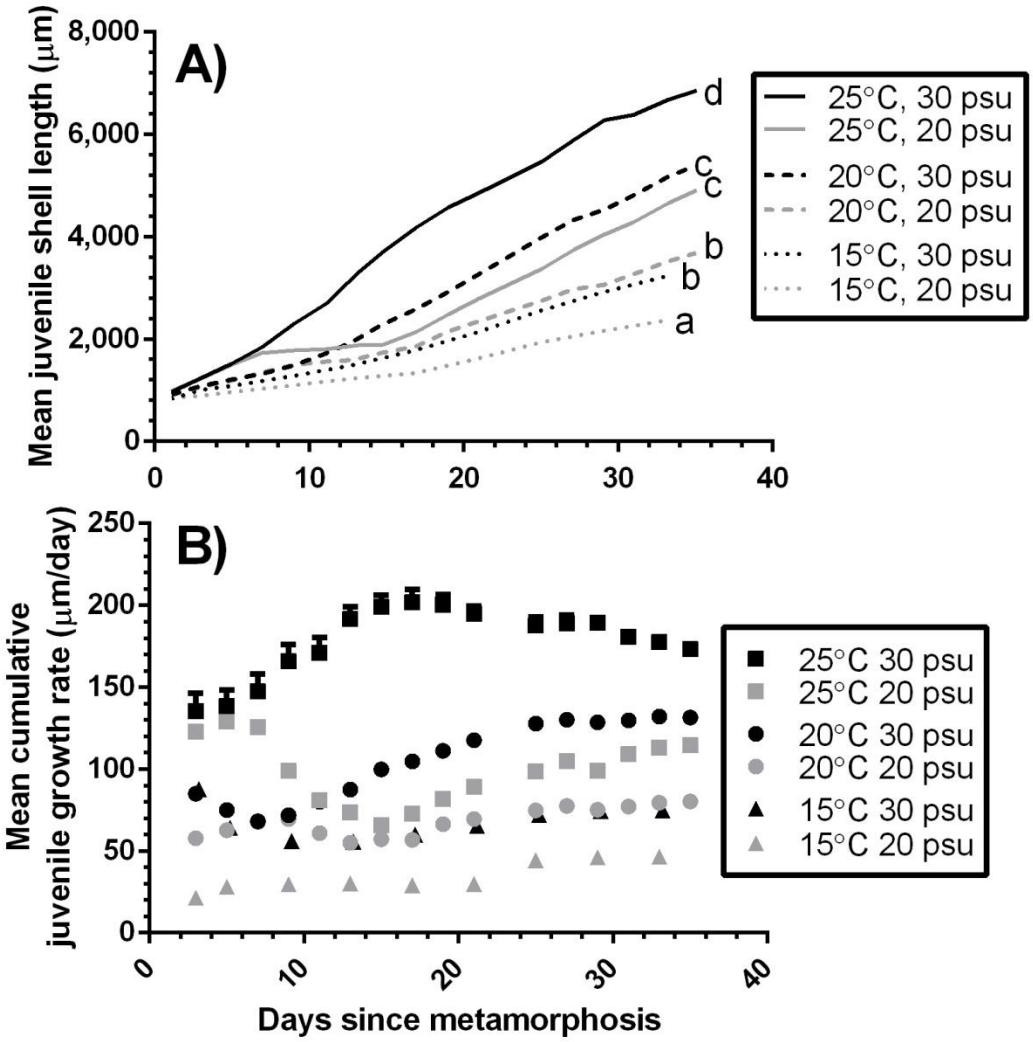

Figure 2. The interactive effects of temperature and salinity on A) shell length and B) mean cumulative growth rates of *C. fornicata* juveniles. Larvae had all been reared in the lab at room temperature (~23°C) and full strength seawater. Juveniles were distributed into treatments 1 day after metamorphosis. Juveniles were first measured on day 1 after metamorphosis and then every 2-4 days after that. Each point represents the mean measurement from 10-12 juveniles. A) A 1-way ANOVA followed by a Bonferroni post-hoc test was run on juvenile sizes in each treatment at day 33 (the last day juveniles in all treatments were measured). Different letters indicate significantly different means ($p<0.05$). B) The standard error plotted on the 25°C 30 psu treatment is representative of all other treatments.

Larval growth

Higher temperature increased larval growth rates by up to 50% and the effect was significant for all larval hatches (2-way ANOVAs, $F>15.92$, $df=1,8$, $p<0.0040$). For larval hatches #1 and #2 (with the temperature treatments 20 and 25°C, and 25 and 29°C respectively), low salinity significantly decreased shell growth rates by up to 50% (2-way ANOVAs, $F>24.47$, $df=1,8$, $p<0.0011$), but for larval hatch #3 (with the temperature treatments 20 and 25°C), salinity had no effect (2-way ANOVA, $F=0.8937$, $df=1,8$, $p=0.3721$; Table 1). There was a significant interaction of temperature and salinity in larval hatch #2 (2-way ANOVA $F=10.98$, $df=1,8$, $p=0.0106$), indicating that salinity had a stronger effect on larval growth at the higher temperature. The interaction was also significant for larval hatch #3 (2-way ANOVA, $F=17.73$, $df=1,8$, $p=0.0030$) because low salinity depressed larval growth at 20°C but increased growth at 25°C (Fig. 3, Table 1).

Abnormal larval development was observed in the 20°C 20 psu treatment of larval hatch #1, but no abnormal development was observed in larvae from this hatch in other treatments. These larvae grew shells that curled upwards around the brim, resembling a bowler hat. Generally, the body could not fit inside the abnormal shell.

Juvenile growth

In all 6 experiments, juveniles reared in low salinity grew up to 75% more slowly, and the effect was significant (2-way ANOVAs, $F>20.09$, $df=1,38\text{-}45$, $p<0.0001$). In 4 out of 6 experiments (20°C hatch #1 and #3, 25°C hatch #2 and #3), exposing larvae to low salinity significantly affected juvenile growth rates (2-way ANOVAs, $F>4.687$, $df=1,38\text{-}45$, $p<0.0357$). For both 20°C experiments and the hatch #3, 25°C experiment, larval exposure to low salinity decreased juvenile growth rates in both juvenile salinities. For the 25°C hatch #2 experiment, the

interaction of juvenile and larval salinities was significant (2-way ANOVA, F=8.401, df=1,39, p=0.0061); larval exposure to low salinity increased juvenile growth rates in low salinity, but had no effect on juvenile growth rates in normal salinity (Fig. 4, Table 1).

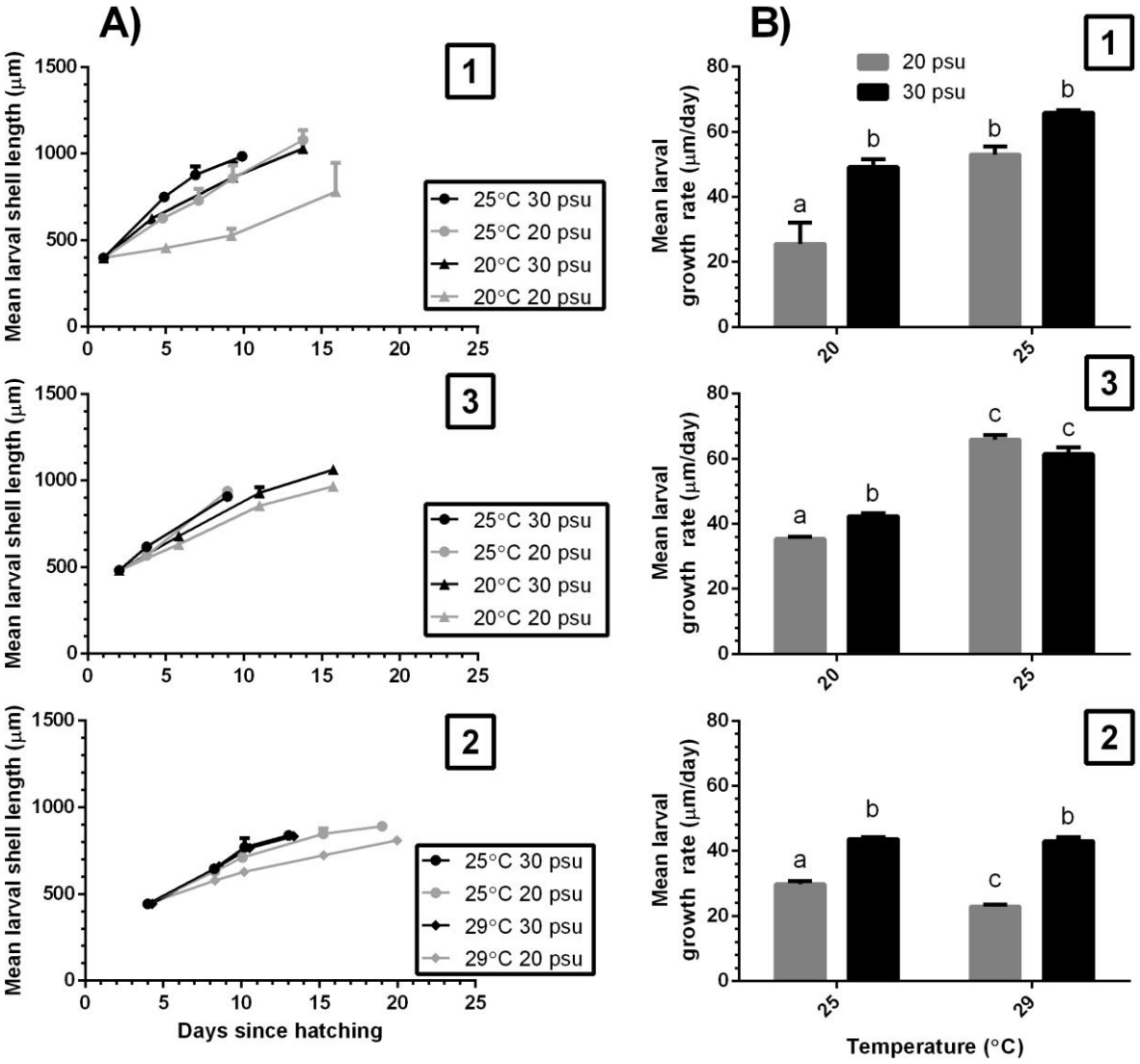

Figure 3. The effects of salinity and temperature on larval *C. fornicata* A) shell lengths and B) growth rates. A total of 3 larval hatches were used in these experiments and the boxed number in each graph indicates which larval hatch those larvae came from. Shell lengths were determined at the start of the experiment and every few days after until larvae reached metamorphic competence. Growth rates were calculated as changes in shell length from the initial measurement to the day of metamorphosis. The 29°C treatments were not repeated but the 20°C treatments were repeated once and the 25°C treatments were repeated twice. Data points or bars indicate the mean of 3 replicate bowls with 15 larvae per bowl and error bars show standard error.

Relative inorganic content

Larvae

Salinity, but not temperature, had a significant effect on larval percent inorganic content (2-way ANOVA, $F=9.585$, $df=1,39$, $p=0.0036$). At 20°C, low salinity depressed inorganic content by 27% relative to control larvae, but salinity had no effect at 25°C so the interaction of salinity and temperature was significant (2-way ANOVA, $F=32.02$, $df=1,39$, $p<0.0001$; Fig. 5, Table 1).

Juveniles

For juveniles from juvenile growth experiment 1, temperature, but not salinity, significantly affected juvenile percent inorganic content (2-way ANOVA, $F=17.48$, $df=2,53$, $p<0.0001$). In low salinity, juveniles had a higher percent inorganic content at higher temperatures. However, at normal salinity the order of increasing juvenile percent inorganic content by temperature was 25°C>15°C>20°C. Therefore, the interaction of salinity and temperature was significant (2-way ANOVA, $F=4.916$, $df=2,53$, $p=0.0110$; Fig. 6, Table 1).

For juveniles from juvenile growth experiment 2, the salinity in which larvae were reared significantly affected juvenile relative inorganic content only in 1 of 6 experiments (25°C hatch #1). In this experiment, larval exposure to low salinity depressed juvenile inorganic content by about 2.5%. The salinity in which juveniles were reared significantly affected juvenile inorganic content in 3 experiments (29°C hatch #2, 25°C hatch #3, 20°C hatch #3), but the direction of the effect was not consistent (Fig. 7; 2-way ANOVAs, $F>12.72$, $df=1,32-41$, $p<0.0012$). The interaction of larval and juvenile salinities was significant for 2 experiments (20°C hatch #1, 29°C hatch #2; 2-way ANOVAs, $F>5.641$, $df=1,32-33$, $p<0.0237$). All differences in juvenile

relative inorganic content from juvenile growth experiments 1 and 2 were of small magnitude (<6%; Figs. 6, 7, Table 1).

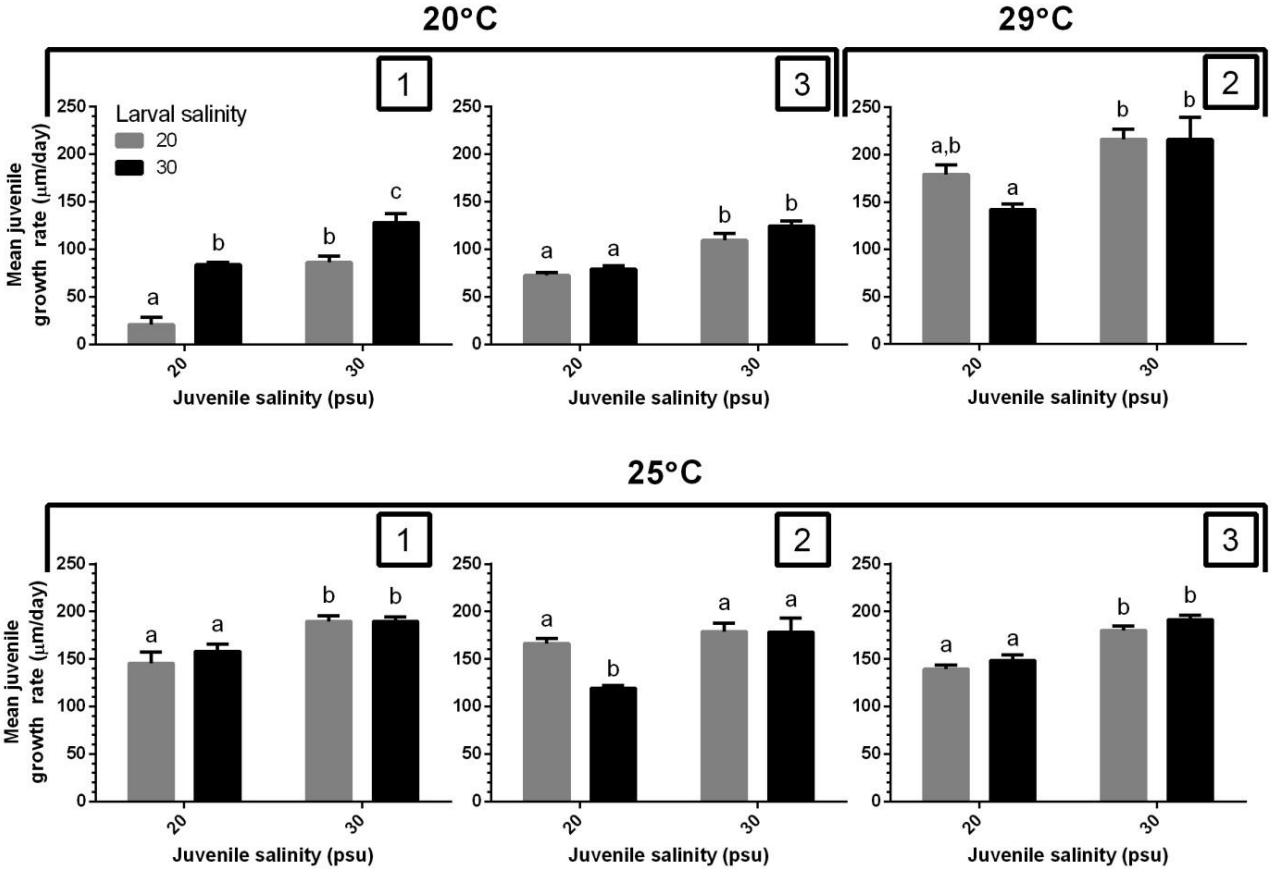

Figure 4. The effects of larval and juvenile rearing salinity on juvenile growth rates of *C. fornicata* in 3 different temperatures. Growth rates were calculated as changes in shell length from day 1 or 2 after metamorphosis to day 16 after metamorphosis. A total of 3 larval hatches were used in these experiments and the boxed number in each graph indicates which larval hatch those juveniles came from. Larvae were reared at either 20 or 30 psu from a few days after hatching until they reached metamorphic competence, when they were induced to metamorphose and distributed among the juvenile treatments. Error bars indicate standard error (n=8-13). Two-way ANOVAs followed by Tukey post-hoc tests were run on the data in each graph and different letters above bars in each graph indicate significant differences ($p<0.05$).

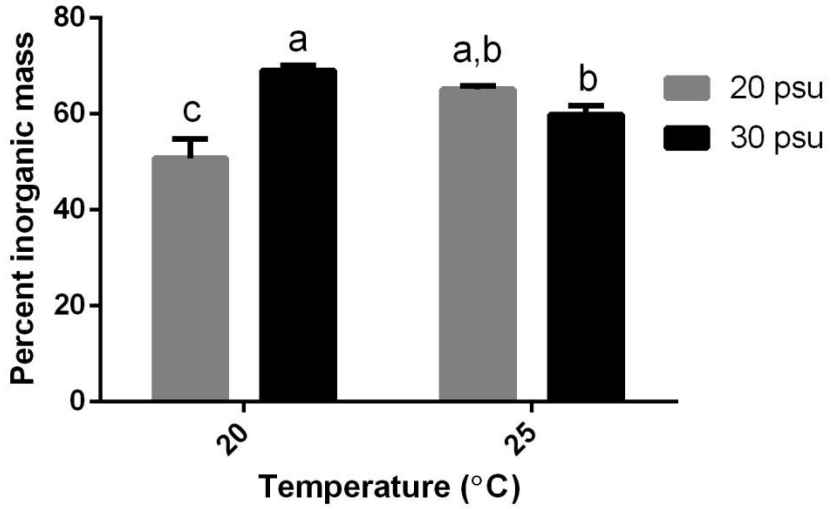

Figure 5. The effects of temperature and salinity on larval *C. fornicata* inorganic content. Larvae were distributed into 4 temperature and salinity treatments 2 days after hatching and sacrificed when they reached metamorphic competence. Inorganic mass was determined by combusting snails at 500°C for at least 12 hours; relative inorganic content was then calculated by dividing inorganic mass by total dry mass. A 2-way ANOVA followed by a Tukey post-hoc test was run on these data and different letters above bars indicate significant differences between means ($p<0.05$). Each bar represents the mean value of 11 samples with 5-9 larvae per sample and error bars indicate standard error.

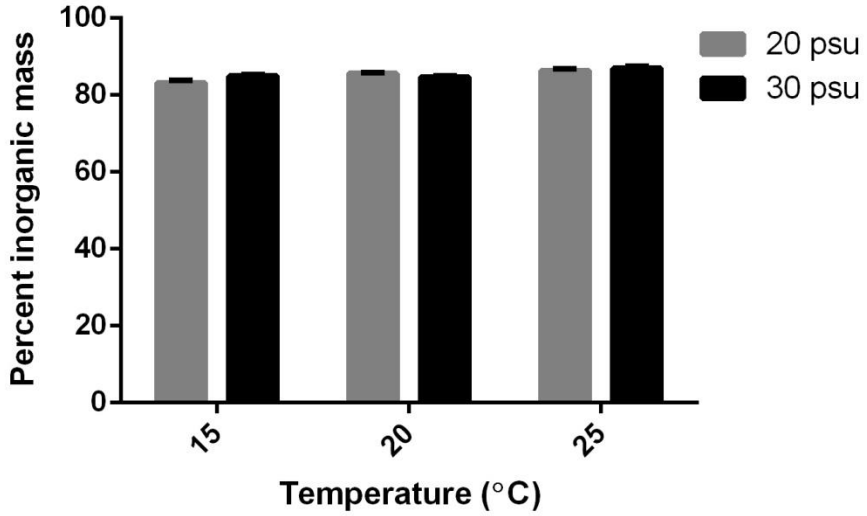

Figure 6. The interactive effects of temperature and salinity on juvenile *C. fornicata* inorganic content. Larvae had all been reared in the lab at room temperature (~23°C) and full strength seawater. Juveniles were distributed into treatments 1 day after metamorphosis. Inorganic mass was determined by combusting snails at 500°C for at least 12 hours; relative inorganic content was then calculated by dividing inorganic mass by total dry mass. All juveniles were approximately the same length (~6-6.5mm) when sacrificed for these measurements. Error bars indicate standard error.

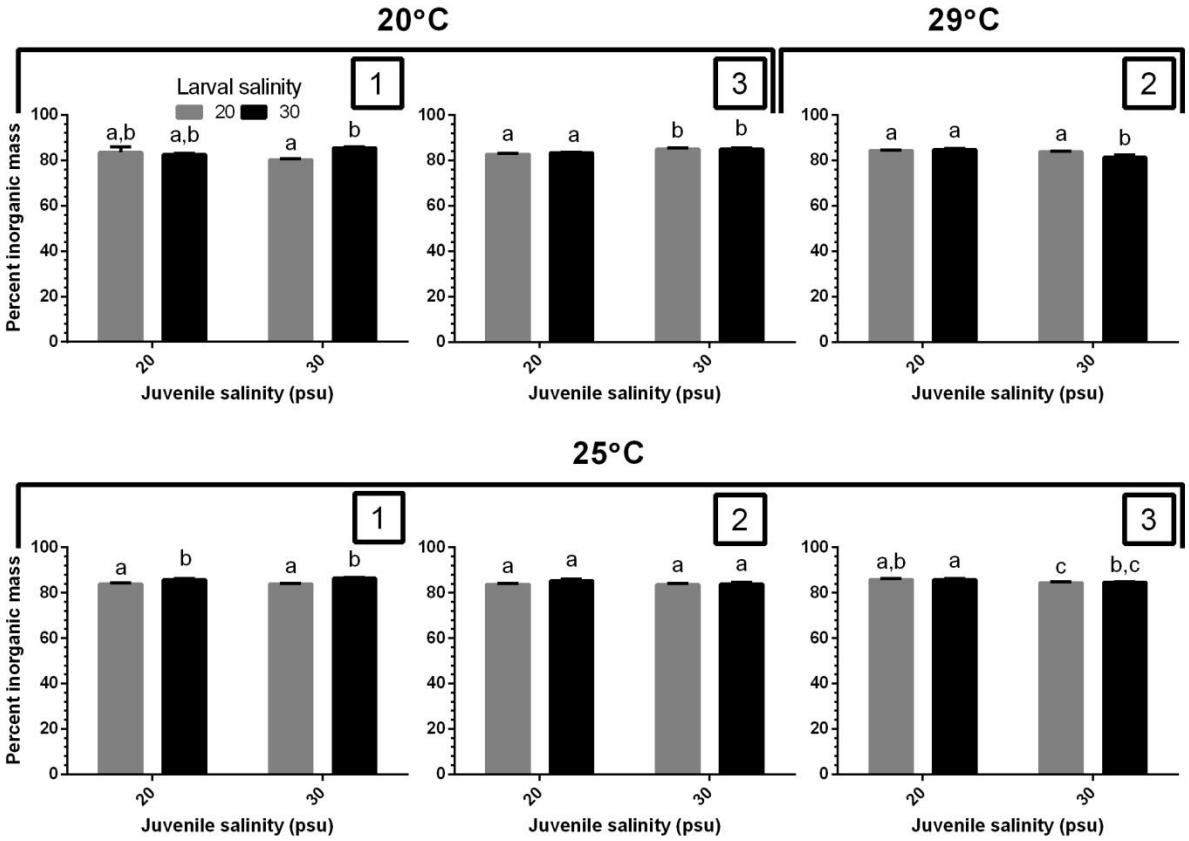

Figure 7. The effect of larval and juvenile rearing salinity on relative inorganic content of *C. fornicata* juveniles at 3 temperatures. A total of 3 larval hatches were used in these experiments and the boxed number in each graph indicates which larval hatch those juveniles came from. Inorganic mass was determined by combusting snails at 500°C for at least 12 hours; relative inorganic content was then calculated by dividing inorganic mass by total dry mass. All juveniles were approximately the same length (~4.5mm) when sacrificed for these measurements. Larvae were reared at either 20 or 30 psu from a few days after hatching until they reached metamorphic competence, when they were induced to metamorphose and distributed into juvenile treatments. Error bars indicated standard error (n=5-13). Two-way ANOVAs followed by Tukey post-hoc tests were run on the data in each graph and different letters above bars in each graph indicate significant differences ($p<0.05$).

Juvenile feeding rates

Juveniles reared in 20 psu were 1542.8 ± 236.4 µm (mean ± SD) long at the time of the experiment while those reared in 30 psu were 1878.0 ± 211.5 µm long at the time of the experiment. For both experiments, there was no significant difference in juvenile shell length between juveniles feeding in 20 psu and those feeding in 30 psu (2 t-tests, $t<0.4905$, $df=32$, $p>0.6$). Juveniles reared in 30 psu fed in 20 psu at 50% the rate they fed in 30 psu and the

difference was significant (t-test, t=4.069, df=32, p=0.0003). For juveniles reared in 20 psu, there was no significant difference between their feeding rates in 20 and 30 psu (t-test, t=1.408, df=32, p=0.1689). However, juveniles in 20 psu fed 20% slower on average than those in 30 psu (Fig. 8).

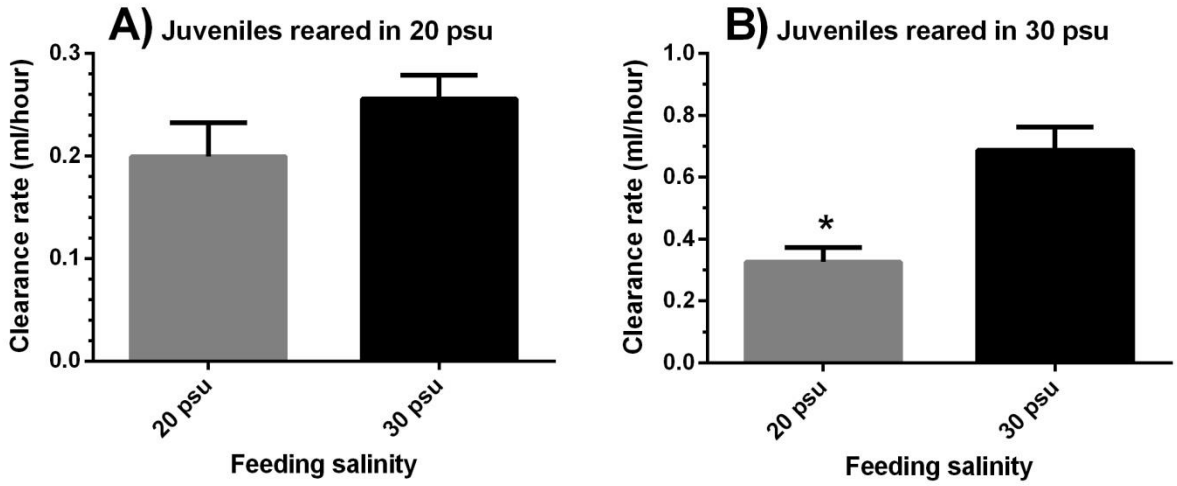

Figure 8. The effect of low salinity on the feeding rates of juveniles reared in either low (20 psu) or control (30 psu) salinity. Larvae reared at room temperature and full strength seawater were induced to metamorphose when competent and the resulting juveniles were then transferred into 2 salinity treatments: A) 20 or B) 30 psu and maintained at that salinity for 20-26 days until experiments were conducted. Juveniles were first pre-fed for 2 hours in the appropriate salinity and then water was changed and clearance rates were determined over the next 2 hours. Juveniles fed in 12-well plates filled with 5 ml of seawater at approximately 1.8×10^5 cells/ml *I. galbana* suspension. Feeding rate was calculated from changes in particle counts as determined with a Coulter Counter. Each bar represents the mean of 17 replicates and error bars indicate standard error. At the time of the experiment, juveniles reared in 20 psu were 1542.8 ± 236.4 µm (mean ± SD) long while those reared in 30 psu were 1878.0 ± 211.5 µm long. There were no significant differences in juvenile size between the 2 treatments in either experiment ($p>0.6$). A "*" indicates a significant difference between mean feeding rates in that experiment ($p<0.05$).

Discussion

These experiments demonstrated that larval exposure to low salinity can influence subsequent juvenile growth in the gastropod *C. fornicata*. However, the magnitude and direction of this influence depended strongly on both temperature and larval hatch (i.e. parentage) and, to a lesser extent, the salinity juveniles were reared in. In addition, juvenile growth rates were

elevated by increased temperature and depressed by low salinity, but the effect was synergistic, such that salinity had a greater effect at higher temperatures.

In the following sections, I will discuss the results of my study in the contexts of 1) larval growth 2) juvenile growth 3) inorganic content 4) latent effects 5) interactive effects 6) mechanisms behind reduced growth 7) hatch variability and then the general 8) implications and 9) conclusions of the study.

1) Larval growth

Larval growth was significantly increased by higher temperatures in the range of 20-25°C in these experiments, agreeing with other studies (Pechenik 1984, Pechenik and Lima 1984, Pechenik and Heyman 1987, Klinzing and Pechenik 2000). In addition, larvae continued to grow, but at a slower rate, in low salinity in 2 out of 3 larval hatches, and at all 3 temperatures: 20, 25, and 29°C. In the other larval hatch (#3), larvae grew significantly more slowly in low salinity than normal salinity at 20°C, but there was no difference at 25°C. The fact that our larvae grew in 20 psu is in contrast to a study by Diederich *et al.* (2011) which found that larvae of *C. fornicata* and 2 closely related species (*Crepipatella fecunda* and *Crepidula onyx*) showed no detectable growth during low salinity stress (10, 15, and 20 psu). While larvae were only exposed to low salinity for 12-48 hours in their experiments, our larvae grew detectibly even in the first 2 days of low salinity exposure (see Fig. 3A, hatch #3). Even in experiments where larvae were not measured within 2 days of low salinity exposure, larvae in low salinity grew at very similar rates to those in normal salinity within the first few days (Fig. 3A), which makes it very unlikely that they stopped growing for the first day or 2 of low salinity exposure. In agreement with our results, Zimmerman and Pechenik (1991) found that larvae of the related species *Crepidula plana* grew significantly more slowly in 20 psu than 30 psu. Studies on other

molluscan larvae have also found reduced growth in low salinity (Richmond and Woodin 1996, Klingston 1974, Scheltema 1965).

While previous studies have observed linear growth in *C. fornicata* larvae (Pechenik 1980, 1984, Pechenik and Lima 1984, Klinzing and Pechenik 2000), we found that this is not always the case. Larvae in a number of treatments grew at different rates over time, especially in the low salinity. Since this is the first study to look at the effects of constant exposure to low salinity on larval *C. fornicata* growth and most larvae growing non-linearly were in low salinity, it is possible that the low salinity exposure was responsible for non-linear growth.

2) Juvenile growth

In the first juvenile growth experiment (when larval salinity was not manipulated), juveniles grew 50-65% faster at higher temperatures (over the range 15-25°C). While no other studies have investigated the effects of temperature on juvenile *C. fornicata*, these results agree with a study on juvenile clams, in which growth rates increased with temperature up to 25°C (Laing *et al*. 1987). However, our results contrast with a study by Thieltges (2004) in which he compared sizes in the field of the first-year cohort of *C. fornicata* from northern and southern climates and found no difference in average estimated growth rate. Also, Zhao (2002) found no significant effect of temperature on growth rates in the lab of juveniles of the closely related species *Crepidula onyx*.

In addition, rearing juveniles in low salinity slowed their growth in all juvenile growth experiments (7 in total), mimicking the larval response to low salinity. Similarly, Diederich *et al.* (2011) found that juvenile *C. fornicata* growth was slowed for 3 days following a short exposure to low salinity (15 psu). However, the authors did not measure juvenile growth rates during that low salinity exposure. Low salinity has been shown to decrease juvenile growth in other species

as well (shrimp: Ponce-Palafox *et al.* 1997, Brito *et al.* 2000, crabs: Cadman and Weinstein 1988, bay scallop: Shriver *et al.* 2002).

3) Inorganic content

Relative inorganic content can serve as a proxy for relative shell weight in these experiments since all samples were rinsed with deionized water before processing, so the vast majority of inorganic matter would be from the shell. In these experiments, larvae and juveniles exhibited a range of responses to temperature and salinity in terms of their relative shell growth.

While juvenile relative inorganic content was significantly affected by temperature and, in 3 of the 7 experiments, by the salinity at which juveniles were reared, the directions of the effects were not consistent and the biological significance is not clear. Differences were in the range of 3-4%, which seems very low, but there are few studies correlating organismal inorganic content to function. A recent study on juvenile oysters found that juveniles reared in 15 psu had significantly lower tissue mass (by 10%) than controls reared in 30 psu but overall mass and relative shell mass were not affected (Dickinson *et al.* 2012). In addition, the shells of these juvenile oysters reared in low salinity tended to show lower fracture resistance than those of controls but the difference was not significant. Since shells were the same mass in both salinity treatments, the quality of newly deposited shell must have differed to cause the trend of decreased fracture resistance. However, this would not have been picked up by the methods of our study unless that difference in quality was due to altered organic content of the shell, which is one of the hypotheses proposed by Dickenson *et al.* (2012). In another study, Dickenson *et al.* (2013) found that juvenile clams reared in 16 psu had 25% lower shell mass than those reared in 32 psu, but tissue mass was also reduced by about 40% so it is likely that the clams were just smaller in low salinity. Also, shells of juveniles reared in low salinity tended to be more easily

fractured, but the difference was not significant. Since a 25% reduction in shell mass had very little influence on the mechanical properties of the clam shells in this study, it is unlikely that the differences observed in our studies are biologically relevant. However, it is very difficult to compare this study to our results since Dickenson *et al.* measured total shell mass rather than relative inorganic content. Therefore, the differences we observed may be more relevant from an energy budget and energy partitioning perspective than in terms of shell protection. However, future studies should investigate the correlation between organismal inorganic content, shell strength, and effectiveness of shell protection from predation and abiotic stresses in *C. fornicata* in order to establish standards of relevance for this easily measured relationship.

Larval inorganic content was strongly influenced by salinity, but not temperature. Inorganic content was reduced by 27% in low salinity, but only at the low temperature (20°C). This is a very large difference in inorganic content and likely to have biological significance. In addition, it could explain the abnormal development observed in larvae from hatch #1 reared at 20°C, 20 psu. If tissue growth were outpacing shell growth in these larvae, the oversized tissue could have exerted too much pressure on the relatively weak shell, forcing it to "pop" upwards into a bowler hat shape. Kingston (1974) also observed deformed development in calcifying molluscan (clam) larvae at 20 psu, but the deformation was not described nor an explanation proposed.

While a number of studies have investigated the effects of reduced salinity on adult growth in terms of shell mass (Brown and Hartwick 1988, Almada-Villela 1984, Nagarajan *et al.* 2006, Malone and Dodd 1967), the effects on larval or juvenile shell mass have been little studied. Since larval and juvenile shells are much smaller and more vulnerable, it is during these stages that organisms are adding functional protection at the greatest rate. Thus, more research

should focus on the effects of environmental factors on larval and juvenile shell growth to better understand this critical period of shell growth.

4) Latent effects of larval exposure to low salinity

It is becoming increasingly clear that transitions to new life stages are not a new beginning but rather that each successive life stage is strongly influenced by events and influences that acted on previous stages (reviewed by Pechenik 2006). The results of our experiments provide further support for this theory.

Juvenile growth was significantly affected by larval exposure to low salinity in 4 out of 6 of our experiments, when larvae and juveniles were reared at 20 and 25°C. In 3 of these experiments, the effect of the salinity in which larvae were reared on juvenile growth was the same regardless of the salinity juveniles were currently living in, which indicates that a change in salinity from larval to juvenile stage did not affect growth, i.e. there was no extra stress detected in these experiments from a salinity change between life stages. In addition, the salinity in which larvae were reared had a significant effect on juvenile inorganic content in only 1 of the 6 experiments, but the magnitude was so small (3%) that it is unlikely to be biologically significant.

Therefore, we conclude that low salinity can cause latent effects in *C. fornicata* juvenile growth rates, in opposition to the results of Diederich *et al.* (2011) which found no latent effects of low salinity. However, there are a number of differences between the two studies. Diederich *et al.* only reared juveniles in stress-free, normal salinity conditions while we reared juveniles in both normal and low salinity. Yet we detected latent effects in both juvenile salinities so this would not make a difference. Also, Diederich *et al*. only exposed larvae to low salinity for 12-48 hours while we reared larvae from 1-4 days after hatching until metamorphosis in the low

salinity. In addition, Diederich *et al.* measured juvenile growth rates over the 4 days following metamorphosis while we measured growth rates from day 1-2 after metamorphosis until day 16 after metamorphosis. It is possible that either A) latent effects do not manifest themselves unless larvae are exposed to low salinity for greater than 48 hours or B) latent effects do not manifest themselves until more than 4 days after metamorphosis. However, we detected latent effects within the first 6 days of juvenile growth (data not published here) so option B is not very likely unless latent effects always appear between days 4 and 6 after metamorphosis with improbably rigidity. Thus, it seems likely that larvae must experience a certain level of salinity stress before latent effects will become apparent in the juveniles. This is further supported by the fact that Diederich *et al.* (2011) did find latent effects in one experiment, but only when larvae were exposed to the very low salinity of 10 psu for 2 days.

Similarly, latent effects of larval exposure to low salinity have been documented in the polychaete *Capitella teleta* (Pechenik *et al.* 2001) and the barnacle *Balanus amphitrite* (Thiyagarajan *et al.* 2007). Adding the results of our study to these previously documented cases of salinity-induced latent effects lends weight to the necessity of considering the environmental conditions larvae are exposed to when attempting to predict the responses of juvenile or adult populations. Juveniles in stress-free conditions would be expected by conventional methods to exhibit normal growth and mortality rates. However, if they were exposed as larvae to low salinity during their dispersal, these juveniles could grow much slower than expected, which would likely increase mortality by predation or abiotic stresses (Vermeij 1972, Paine 1976), thereby limiting the supply of adults in the future.

5) <u>Interactions between environmental factors</u>

Stresses experienced by organisms due to changing environmental conditions will not be felt in isolation. Therefore, it is very important to study the interactive effects of environmental variables. In this study we found a number of significant interactive effects of salinity and temperature on larval and juvenile growth and inorganic content.

In 2 out of 3 larval hatches (#2 and #3), there was an interactive effect of salinity and temperature on larval growth. In larval hatch #2, low salinity depressed larval growth more at the higher temperatures (29 vs. 25°C) while in hatch #3 there was a trend of slower larval growth in low salinity at the lower temperature (20°C) but faster larval growth in low salinity at the higher temperature (25°C). As for juveniles, low salinity had a greater effect on growth at higher temperatures and temperature seemed to modulate the effects of larval exposure to low salinity on juvenile growth. Larval salinity affected juvenile growth rates at 20°C in both experiments, at 25°C in 2 out of the 3 experiments, but not at 29°C. This could suggest that larval exposure to low salinity has a greater effect on juvenile growth at lower temperatures, in contrast to the temperature interaction with the salinity at which juveniles were reared, where low salinity had a greater effect on juvenile growth at lower temperatures. However, since the 29°C experiment was only conducted once, more experiments are needed to lend more support to this hypothesis. Finally, there was a strong interaction between temperature and salinity in larval inorganic content. At the lower temperature (20°C), larvae had a much lower inorganic content at 20 psu than at 30 psu, while at the higher temperature (25°C) there was no effect but a slight trend of greater inorganic content in 20 psu than at 30 psu. Therefore, these experiments demonstrate the importance of temperature not only in its direct effects on growth rates, but also in the way it modulates the effects of other variables such as salinity.

Previous studies have likewise found significant interactive effects of temperature and salinity on the growth and mortality of marine organisms (larval clams and oysters: Calabrese and Davis 1970, oysters: Heilmayer *et al*. 2008, Hutchinson and Hawkins 1992, embryonic and larval mussels: Verween *et al.* 2007, juvenile shrimp: Ponce-Palafox *et al.* 1997, snail embryos: Przeslawski *et al.* 2005, juvenile snails: Berry and Hunt 1980, juvenile crabs: Brown and Bert 1993, juvenile sea stars: Chen and Chen 1993). Two main types of temperature-salinity interactions were observed in these studies: 1) Six studies found that organisms were more resistant to the effect of low salinity at low temperatures (Heilmeyer *et al.* 2008, Hutchinson and Hawkins 1992, Ponce-Palafox *et al*. 1997, Berry and Hunt 1980, Brown and Bert 1993, and Chen and Chen 1993) and 2) two studies found that organisms were more tolerant of high salinity at low temperatures and vice versa (Przeslawski *et al.* 2005, Verween *et al.* 2007). While we did not test the effects of high salinities, we did find examples of both types of temperature-salinity interactions. The interaction of juvenile exposure to low salinity with temperature conformed to the first type of interaction, where juveniles were less affected by low salinity at low temperatures, but larval exposure to low salinity conformed to the second type of interaction, where low salinity exposure as larvae had a smaller effect on juvenile growth at higher temperatures. The interaction of salinity with temperature on larval growth was less clear.

6) <u>Mechanisms of reduced growth</u>

This study has demonstrated the effects of temperature and salinity on the growth of *C. fornicata* larvae and juveniles, the interactions between these factors, and the latent effects of larval low salinity stress on juvenile growth. Therefore, the next question is how and why growth rates are affected by these environmental factors.

Temperature likely increased growth rates by increasing juvenile and larval feeding or assimilation rates. For example, Hutchinson and Hawkins (1992) found that the oyster *Ostrea edulis* fed significantly faster at higher temperatures over the range 5-25°C while Crisp *et al.* (1985) found that oyster larvae fed faster at higher temperatures from 10- 20°C but feeding rate decreased from 20 to 30°C. However, respiration rates (and therefore rates of energy expenditure) generally increase with temperature as well (e.g. juvenile clams: Laing 1987). Since the juveniles and larvae in our experiments grew faster at higher temperatures, it is likely that increases in feeding and/or assimilation rates outpaced any temperature-mediated increases in respiration. Future studies on the effects of temperature on larval and juvenile *C. fornicata* feeding, respiration, and assimilation rates should be conducted to confirm this.

The effects of salinity on larval growth could also be due to changes in feeding, assimilation, or respiration rates. *C. fornicata* larvae in 20 psu fed at 50% the control rate (Diederich *et al.* 2011). However, a study on crab larvae found decreased respiration rates at low salinity, indicating that energy expenditure decreased at low salinities, and reduced assimilation efficiency was discovered to be the primary reason for reduced growth in low salinity in this study (Anger *et al.* 1998). Therefore, it is likely that a combination of reduced feeding rate and assimilation efficiency overcame any decreases in respiration to limit the energy availability and caused our larvae to grow more slowly in low salinity in 2 out of 3 larval hatches.

As for the response of juveniles to low salinity, we found that when juveniles reared in 30 psu were transferred to 20 psu, they fed at 50% the control rate. Also, juveniles reared at low salinity demonstrated a trend of 20% decreased feeding in low salinity but the difference was not significant. In addition, a study on juvenile clams found that low salinity caused standard metabolic rate to increase (Dickinson *et al.* 2013). A combination of decreased feeding rate with

increased energy demand would very likely lead to the decrease in growth rates in low salinity observed in all juvenile growth experiments. However, more research should be conducted on juvenile molluscs to determine to what degree responses to low salinity are similar among species.

The mechanisms behind latent effects are not well understood. Since the stress was experienced in a previous life stage, it is less likely to have the same sort of direct effects that stresses experienced during the juvenile stage have on juvenile growth. However, studies have shown that latent effects can manifest themselves as damages to juvenile feeding structures (Wendt 1998, Marshall *et al.* 2003). Other possibilities could be reduced energy reserves, effects on transcriptional or translational processes, altered DNA methylation, and/or damaged DNA or important enzymes (reviewed by Pechenik 2006).

However, one big difference between the temperatures and salinities tested in this study is that the temperatures used were not particularly stressful and were likely to be experienced by these intertidal snails in their natural habitat, while the low salinity was a large, stressful change that would be experienced only rarely in the natural habitat of *C. fornicata*, perhaps following heavy rains and low-tide exposure or extreme freshwater discharges. This type of osmotic stress could damage the snails and thereby further reduce growth rates in this osmoconforming gastropod. Some molluscs can regulate cell volume in hypoosmotic conditions (Pierce and Amende 1981) by expelling amino acids into extracellular fluid but this could conceivable be an expensive solution since fewer amino acids would be available for protein synthesis within cells.

The differences in relative inorganic content observed in this study could be indicators of energy partitioning in response to unfavorable conditions. If these snails were facing a reduction in energy supply and an increase in energy demand, they may have devoted more of their

resources to growing tissue in order to increase the size of the feeding apparatus and thereby increase the energy supply. The large difference in larval relative inorganic content at 20°C between larvae reared in 20 and 30 psu could be a dramatic example of this, since it has been shown that larval *C. fornicata* feeding rates are significantly reduced at low salinity (Diederich *et al.* 2011). In addition, the smaller differences noted in juvenile relative inorganic content could also be indications of this type of compensation for an energy deficit.

7) Hatch variability

Our experiments demonstrated a wide variability in responses to salinity and temperature and in overall growth rates between larval hatches. For example, larvae reared at 20°C and 20 psu from hatch #1 developed abnormally into a bowler hat shape, while larvae in the same conditions from hatch #3 developed normally and grew 44% faster than those from hatch #1 (Fig. 3B). In addition, juveniles at 25°C and 20 psu actually grew 40% faster after prior exposure to low salinity in one hatch (#2; Fig. 4) but that effect was not observed for juveniles in any other hatches. Previous studies have also documented genetic or heritable differences between larval hatches in *C. fornicata* (Diederich *et al.* 2011, Hilbish *et al.* 1999). The effect of this hatch variability in our experiments seems to indicate that different larval hatches will be differentially affected by temperature and salinity conditions. Some hatches may express latent effects from low salinity exposure during larval development while others will be unaffected (e.g. compare 25°C hatch #1 with hatches #2 and #3 at the same temperature) and some larvae may experience decreased growth rates at low salinity while others will not (larval hatch #3 vs. #1 and #2). This indicates that there could be room for natural selection to act and select for the more hardy individuals as environmental conditions continue to change. In addition, this variability could explain the great success of *C. fornicata* as an invasive species since the large range of

temperature and salinity tolerances within the population could make it easier for the species to invade habitats with a wide range of abiotic conditions. Also, hatch variability in temperature and salinity sensitivity lends more weight to the factors that influenced larval or juvenile growth rates in every single hatch, such as the effect of temperature on larval and juvenile growth and the effect of the salinity in which juveniles were reared on juvenile growth. These factors exerted a big influence that no amount of genetic variation seemed able to compensate for.

8) Implications

These experiments demonstrate the complexity of temperature-salinity effects on *C. fornicata* larvae and juveniles. Temperature and salinity were related with a number of interactive effects and parentage seemed to have a large influence as well. As mentioned in the previous section, the most confident conclusions of this study are that *C. fornicata* larvae and juveniles grew faster at higher temperatures and *C. fornicata* juveniles grew more slowly in low salinity. In addition, prolonged larval exposure to low salinity influenced juvenile growth but the effect may depend on temperature and/or larval hatch. Low salinity decreased larval inorganic content, but only at the low temperature (20°C).

The implications of these results are that in the absence of salinity stress *C. fornicata* may do better as oceanic temperatures continue to increase. Larvae and juveniles will grow faster, and so will be more likely to escape size-selective predation or mortality from abiotic factors (Vermeij 1972, Paine 1976, Rumrill 1990, Pechenik 1999, Morgan 1995), and thereby increase the supply of new adults to the population.

In regions with frequent exposure to low salinity, or where salinity is decreasing due to the altered precipitation patterns or glacial melting caused by global warming (IPCC 2013), *C. fornicata* may not fare as well. Low salinity reduced larval growth rates by as much as 50% in 2

out of 3 larval hatches and reduced juvenile growth rates in all experiments. However, it is possible that juveniles may be able to adapt to low salinity, since in this study we found that juveniles reared in 20 psu fed at statistically similar rates in 20 and 30 psu, but juveniles reared in 30 psu fed much more slowly in 20 psu. Yet juvenile growth rates were reduced by low salinity for as long as 33 days, so if they can adapt to low salinity it must either take a very long time or not be a strong enough benefit to bring growth rates back to normal levels. In addition, juvenile growth rates were affected by larval exposure to low salinity in 4 out of 6 experiments with the largest effect (in juveniles reared at 20 psu and 20°C) causing a 4-fold reduction in growth rates. Also, the relative inorganic content of larvae at competence (~900µm) was reduced in low salinity by 27% at 20°C. This could severely reduce the effectiveness of the larval shell in protecting from predation or environmental stress. Therefore, low salinity exposure will likely have the opposite effect of increasing temperatures. Larvae and juveniles exposed to low salinity will spend more time at smaller sizes and possibly face increased size-selective mortality as a result. Furthermore, larvae may be less likely to reach suitable habitats if they spend abnormally long in the plankton since currents could carry them too far before they become competent to metamorphose. This could severely limit the size of adult populations. Or, increased time in the plankton could increase the dispersal potential of *C. fornicata* and aid in its invasion of new habitats.

However, these experiments show that despite any changes to growth rates, *C. fornicata* can tolerate, grow, and feed in any combinations of the salinities 20 and 30 psu and the temperatures 15-29°C for juveniles and 20-29°C for larvae. The purpose of this study was not to determine the temperature and salinity limits for *C. fornicata* larvae and juveniles, so it is very likely that they can tolerate an even wider range of temperatures and salinities. Note that despite

the negative effects of low salinity on *C. fornicata*, larvae and juveniles can survive, grow, and feed in 20 psu, a 33% drop from the normal level of 30 psu. In fact, *C. fornicata* has been reported to live in estuaries both in its native and invasive ranges (Chipperfield 1951, Blanchard 1977).

Given the conflicting effects of increasing temperature and decreasing salinity on the early life stages of *C. fornicata*, it is difficult to predict how this species will be impacted by changing conditions in the future. The specific effect will likely depend on the salinity and temperature conditions of each habitat. However, this study emphasizes the importance of including larval experience in models attempting to predict the effects of climate change on species distributions. Larval exposure to low salinity can have a significant effect on juvenile growth rates and these effects can persist for at least 16 days.

Beyond *C. fornicata*, these results may indicate that related species will be affected by temperature and salinity in similar manners. Other gastropods or molluscs (many of which are commercially important) may also face latent effects from low salinity exposure as larvae. More research should be conducted on latent effects in other species of molluscs in order to determine how wide-spread these effects are.

Future studies should continue to investigate the effects of environmental change on the early life stages of marine organisms and how stresses experienced in one life stages may carry over to affect later stages. These early life stages are incredibly important in determining the distribution and abundance of adult populations since many adult populations are limited by recruitment, which is a product both of larval supply and juvenile survival (Keough and Downes 1982, Hunt and Scheibling 1997). In addition, environmental factors should be studied in combination since there are often interactive effects between factors. Special focus should be

applied to the mechanisms behind reduced growth in low salinity or the mechanisms behind these observed interactions. If we can understand the mechanisms behind changes in growth rates, it may make it easier to generalize the results of studies such as this to more species.

9) Conclusion

In conclusion, the growth rates of *C. fornicata* larvae and juveniles were directly related to temperature and salinity; moreover, exposure to low salinity as larvae generally reduced juvenile growth rates (except in the one case it increased juvenile growth rates), juveniles fed more slowly in low salinity, and the effects of salinity were modified both by temperature and by parentage. These experiments demonstrate the complex effects environmental change will have on marine organisms and the importance of more research in this area to fully understand the potentially devastating effects of climate change acting through the poorly understand early life stages of marine invertebrates.

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